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(54) Title: HUMAN CELL CYCLE CHECKPOINT GENES

(57) Abstract

Human checkpoint *huCDC34*, *huRAD9*$_{compA}$, and *huRAD9*$_{compB}$ cDNAs are shown in Figures 1, 2 and 3. A method for isolating a human checkpoint cDNA that is capable of restoring growth at a restrictive temperature in a yeast test cell, wherein the yeast test cell comprises a genome having a first gene that forms a DNA strand break at a restrictive temperature and a second gene that fails to induce a cell cycle arrest in response to the DNA strand break, whereby the growth of the yeast test cell is inhibited at the restrictive temperature, the method comprising the steps of: obtaining a human cDNA library comprising a plurality of human cDNA clones; inserting the human cDNA clones individually into plasmid vectors comprising a selectable marker gene; transforming a culture of the yeast test cells with the plasmid vectors from the preceding step; selecting for yeast test cells transformed with the selectable marker gene; growing the selected transformants at the restrictive temperature and isolating a candidate transformant capable of growing at the restrictive temperature; and identifying the human cDNA carried by the candidate transformant as a human checkpoint cDNA by sequencing the human cDNA carried by the candidate transformant and determining that the human cDNA is less than 50 % homologous with both the first gene and the second gene.

huRAD9$_{compB}$ sequence

ATTGCAGGTTCCGTAGCTTTCTAGTTTTTTTTTTTTTTTCACTTGGATCAAATAGTT
TTGATAGACAGAAAAAGATCTGTACCATTATTTCCTTTCCTTAACAGCTATTGTAA
TTTCCTGGACTTGGTTGCTTTTCACTTGGGCAGTTAAGAAGACACAGCTTGTTTTC
CCCATCAGTTTTCTCTCTCTCCTTCGTGTGTGTGTGTGTGTGTGTGTGTGTGTG
TGTGTGTGTGCGTGCGTGCACAGGGCCAATCTTCAGGCTTATGGCTTTTGGAA
CATTTTCTTAATTTAATAGAGAACAGAATTCAATGATTAGCAACATCACTAAAAA
TTTACCCCATTTCTTCTCCATGAGTCACTGACACCCGATGCGCATGAACAGTCCAA
CGTCCACCTCGTAAGATGTCATCGGGGTTCAGGGTTCAGAAGCATCGAGGACTGG
TGGCCGGCCCTCTGTGCTCGCCGTGTGACAATTCCAGTGGCTTTCCTGGCACCATC
AGATGCCTGGTGCCACAAGCTTGGGTCTGCTCCTAGGGGGACGAGGGGTTCCTCC
TCCTCCTCAATTGCTTTATGTGCCTTCACTCAGTGAACCCCAATGGGATGGACAAC
CTGACTTTTTAAACCTAAGGGTTGGGCCTGAACGATGATTACTTTGCCCACGTGC
CTTCTAGGTGCCGAATGTGTGTTCCTGTGATATTGACGTTGACATCCCTGCGGATT
CAGCCACAGGTTTCTGACAAGCTGGAGGAAGCAATGGTAATTTTGGCTTTTTCGG
TTTTGTCTTCAGATAATGAAAAGCTTTTGTAAAACAGCTGAGTGTCAATATGAGTT
CTATGGCTTCAATCTCCTTTAAAAATAAAATTCTTAAGGGTCCAAAACAAAGAAG
AGGGGGCAAATTAAAACCCAATAAAAGGAAAAGAAAAGAAAGAAAACCAAACCC
CAAACAAGAAAAAAGAAAAAAAATTGCTGATATTGCCACAAATCATTAGAAATCT
CCTGACATGCTGAAACCAAATGGTCGTAAGTTCAAAACAAATCAGTGACTTGTTT
TTAATTTTTTGTGGTTTCCTTTTGCTCTTTCTGCCCCTTTGCCGTCCGATTGGTGAT
GTTATTCAAACAGGACCGGATCCCTGCTAAGTGCAGGAGGGACCCTGCCGCTTCT
TTCATCTCCTCATCATCGCTCTCGGGGGGCTTTTCGGTGCGTCTCTTTTTGAGGGG
CAGTGTGTCGCTGGGGACCTTCCTGGCCTTGGCGAAGTGCTGGCGCTTCTTGTGC
TGGGATGCGTACCCGCTGTCCCCCAGAGAATCCTTGGGCTCCTTCTGGCTGTGCT
TCCTGTCGTCCTCTTCCGTGTCGCTGGGGCTCTCGTGGCTCCGGAAGCTCCCCTCG
CTGCCCTCGCTGCCCTCCTGGCTCCCCTTGGTGGCAAACTCATAGTGGTCGTCGGC
TGAGGAGGAGGAGGAGGAGATGGAGTCGCTGGTGGGCGAGGTGCTCCGGGCGTT
GGAGGACTTGGCACTGCTGTAGTTGTGATCCTCCTTGGGGTCTCCGCTGACCACT
GGGGAGCCACAAGATGGCTCACTCTCAGTCCGCATCCGGCAGCTGGTGATGCCAT
TCCTCATGGCCGCTGTCACCCCAATGGGAGTGATTGGCA

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HUMAN CELL CYCLE CHECKPOINT GENES

This application is a continuation-in-part of Serial No. 07/884,426, filed May 14, 1992, which is a continuation-in-part of Serial No. 07/882,051, filed May 12, 1992.

This invention was made with government support under grant GM17709 awarded by the National Institutes of Health and grant CA57156 awarded by the National Cancer Institute. The government has certain rights in the invention.

Field of the Invention

The invention relates generally to molecular biology, genetic engineering, and recombinant techniques, and specifically to checkpoint genes and proteins and surveillance mechanisms for controlling timing of the cell cycle prior to mitosis so that DNA damage by radiation, chemicals, or drugs can be repaired.

Background of the Invention

Cellular resistance to DNA damage and replication errors is critical to survival of cells, tissues, and organisms. Radiation induces DNA strand breaks. Failure to repair even one DNA strand break can be lethal in yeasts. Cellular resistance to DNA damage consists of separate processes for recognition of damage and repair. Control mechanisms exist for arresting the cell division cycle (cdc) until DNA repair is completed. Delay can occur in different phases of the cell cycle depending on the type of DNA damage and the stage in the cell cycle at which the damage occurs. In particular, damage resulting from DNA strand breaks caused by ionizing radiation or topoisomerase inhibitors causes delay of the cell cycle in the G2 phase before entry into mitosis. The delay may be observed as a decline in the mitotic index of human or yeast cells approximately one hour post irradiation.

Several classes of mutations in yeasts have been defined that result in deregulation of the cell cycle. Temperature-sensitive (ts) mutations in yeast cdc genes can result in death at defined points in the cell cycle when strains are shifted to the non-permissive temperature, and lethality may increase in a temperature-sensitive manner (1). More than thirty-two different cdc genes have been identified in *S. cerevisiae* (2). One such mutant, *cdc9-8*^{ts}, is a DNA ligase mutant in which the temperature-dependent increase in lethality presumably occurs because of a general failure in ligating chromosomal DNA Okazaki fragments following chromosomal DNA replication. The molecular activities of most cdc genes is largely unknown.

Recently a new class of cell cycle regulatory mutations has been identified and labeled checkpoint mutations (3). Checkpoints exist to ensure that DNA synthesis is completed before mitosis begins; that anaphase is delayed until all the chromosomes arrive on the metaphase plate; that centrosome duplication does not occur until DNA has been synthesized; and, that initiation of DNA synthesis is coordinated between different regions in a chromosome. In yeast, *RAD9* is one such checkpoint gene of *S. cerevisiae* that mediates G2 delay after DNA damage. *rad9* mutants have greatly increased radiation sensitivity (less than 0.1% survival at 8000 rads for *rad9* yeasts vs. 30% for *RAD+* yeasts) (4). Direct visualization of budding yeasts after irradiation shows that *rad9* cells continue into mitosis despite potentially lethal DNA damage and die in subsequent generations. RAD9 protein is not required for DNA repair, and *RAD9* is not an essential gene in the cell cycle. In the absence of DNA damage, *rad9* cells display normal cell cycle kinetics but accumulate spontaneous chromosome loss at a higher rate than wild-type strains. Northern blot analyses of RNA from yeast in different parts of the cell cycle and from pre- and post-irradiated cells show a constant level of *RAD9* mRNA. The yeast *RAD9* gene has been cloned, and the translated open reading frame encodes 1309 amino acids that exhibit no significant homology to any other known proteins in the database (4). No human genes have been identified that mediate the G2 delay induced by DNA damage.

The simultaneous presence of both a *rad9* checkpoint mutation and a *cdc9-8* mutation (i.e., in a double mutant strain) substantially increases the rate of cell death when cells are shifted to the nonpermissive temperature (4). This increase in lethality is presumably due to DNA strand breaks resulting from incomplete DNA synthesis (*cdc9-8*) and failure to properly delay the cycle to repair the damage (*rad9*).

CDC34 (not to be confused with p34^{cdc2}) is an essential gene in yeast required for the transition from late G1 to the initiation of DNA synthesis (5).

Sequence analysis and enzymatic assays support the notion that CDC34 is an E2 ubiquitin ligase. The target protein ubiquitinated by CDC34 is unknown.

While it has been possible to study checkpoint genes in yeast, few of their human counterparts have been identified and it is not presently known whether events observed in yeast will be generally applicable to cell cycles of higher eukaryotes.

Summary of the Invention

A genetic protocol is disclosed to identify human checkpoint cDNAs based on increased lethality of yeast mutants having a temperature-sensitive mutation that produces damaged DNA and a checkpoint gene mutation that hinders DNA repair. The strategy utilizes the *cdc9-8* yeast strain with a DNA ligase mutation (temperature sensitive for DNA damage), and the *mec1* or *rad9* checkpoint mutations (impaired in G2 arrest and thereby in DNA repair).

The subject screening assay uses the double mutants *mec-1,cdc9-8* (ATCC No. 74155) and *rad9,cdc9-8* (ATCC No. 74154) as yeast test cells to select and isolate human checkpoint cDNA clones that are capable of complementing or suppressing a defective yeast G2 checkpoint function. Feasibility of the assay was established in experiments that identified three novel human genes involved in human cell cycle control: *huCDC34*, $huRAD9_{compA}$, and $huRAD9_{compB}$.

The *huCDC34* cDNA clone (clone #1; alias *171tx61*) was identified as suppressing the *mec-1* checkpoint mutation that renders *mec1,cdc9* cells temperature sensitive for growth at 30°C. Clone #1 did not suppress the checkpoint defect in *rad9,cdc9-8*. The nucleotide sequence of clone #1 is shown in FIGURE 1. Sequence analysis revealed a surprising homology between the isolated human cDNA and the previously cloned *CDC34* gene of *S. cerevisiae*. (Yeast *CDC34* is a member of the E2 ubiquitin ligase family and has no significant homology with *MEC1*.) Human clone #1 cDNA complemented a $cdc34^{ts}$ yeast mutation, confirming its identity as a human homolog of yeast *CDC34*. The *huCDC34* gene is expressed in multiple cell lines, and Southern blot analysis reveals evidence for a single gene that is highly conserved in higher eukaryotes. The *huCDC34* gene was mapped to a telomeric region of chromosome 19p13.3, and the mouse *CDC34* gene mapped to chromosome 11. The position of the *huCDC34* gene in the genome has not been preserved during evolution, designating a novel region of synteny in chromosome 19.

The $huRAD9_{compA}$ cDNA clone (clone #2; alias *83tx42*) was identified as suppressing the *rad9* checkpoint mutation that renders *rad9,cdc9* cells temperature sensitive for growth at 30°C. Clone #2 suppressed the checkpoint defect in *rad9,cdc9-8* as well as *mec1,cdc9-8* cells, but failed to complement the defect in the

cdc9-8 cells. The nucleotide sequence of clone #2 is shown in FIGURE 2. The long open reading frame (ORF) in clone #2 had no significant homology to any previously described protein. The phenotype of *huRAD9*$_{compA}$ appears to be a slowing of the cell cycle during S phase to allow more time for DNA repair.

The *huRAD9*$_{compB}$ cDNA clone (clone #3; alias *171tx23*) was identified as suppressing the *mec-1* checkpoint mutation that renders *mec-1,cdc9* cells temperature sensitive for growth at 30°C. Clone #3 suppressed the checkpoint defect in *rad9,cdc9-8* or *mec1,cdc9-8* cells, but not in *cdc9-8* cells. Clone #3 was capable of conferring radiation resistance upon a single mutant *mec1* or *rad9* cell. The nucleotide sequence of clone #3 is shown in FIGURE 3. The ORF in clone #3 had no significant homology to any previously described protein. The phenotype of clone #3 suggests that it may act in a G2 arrest pathway that is either downstream of, or independent from, both RAD9 and MEC1.

These results demonstrate the success of the subject protocol for selecting and isolating novel human cDNAs that are active in regulating the human cell division cycle.

Brief Description of the Drawings

FIGURES 1, 2, and 3 depict the disclosed *huCDC34*, *huRAD9*$_{compA}$, and *huRAD9*$_{compB}$ cDNAS, respectively.

Detailed Description of the Preferred Embodiment

Methods are disclosed for selecting and isolating human cDNAs responsible for radiation sensitivity and resistance. The methods are based on selecting human cDNAs that complement or suppress checkpoint mutations in yeast cells. The strategy involves using yeast double mutants that are: 1) conditional for DNA damage (e.g., as the result of a DNA ligase mutation); and, 2) mutant for a cell cycle checkpoint function that is necessary to repair damaged DNA (e.g., a G2 checkpoint where arrest allows DNA repair).

Prior to conducting the experiments described below, it was not known whether human checkpoint genes could exert their actions in yeast. While certain other *cdc* genes had been shown to be capable of crossing over between yeast and man, it was reasoned that critical checkpoint functions could be species specific and highly evolved to fit the functions of a yeast or human cell. Expression of a human checkpoint cDNA in a double mutant yeast cell might either go unnoticed, because the human protein could be incapable of rescuing the double lethal mutation, or it might even prove lethal when in a yeast background. Considering the improbable nature of any effect, a screen assay was developed for screening hundreds of

thousands of transformants. Remarkably, in the first series of experiments, a single active clone (clone #1) was identified out of a total of 200,000 transformants. Sequence analysis revealed significant homology of the human cDNA to the *CDC34* gene of *S. cerevisiae*. Clone #1 human cDNA efficiently complements a yeast *cdc34* mutation, identifying it as a human homolog of yeast *CDC34*. The result of this assay was particularly unexpected since all previous reports linked the function of yeast *CDC34* to events in the late G1 phase of the cell cycle prior to entry into S phase. Interestingly, the limited sequence homology between yeast and man was not sufficient for yeast cDNA to identify human CDC34. (S. Plon, unpublished.)

As used herein, "checkpoint" is intended to mean a timepoint in the cell cycle of a eukaryotic cell at which progression to mitosis may be arrested if the cell contains chromosomal DNA with one or more strand breaks. Illustrative methods by which DNA strand breaks may be introduced into chromosomal DNA include DNA ligase mutations, topoisomerase mutations, X-irradiation, gamma-irradiation, and treatment with drugs (e.g., hydroxyurea), or treatment with chemotherapeutic agents, e.g., 5-fluorouracil, ectopside, and the like.

"Checkpoint gene" is intended to mean a gene whose expression (i.e., as RNA or protein) is capable of arresting a cell cycle at a checkpoint in a eukaryotic cell having chromosomal DNA with one or more strand breaks, but not in a eukaryotic cell having native chromosomal DNA, i.e., without DNA strand breaks. The checkpoint gene is capable of conferring upon a eukaryotic cell increased capacity to protect against potentially lethal damage, meaning that the subject eukaryotic cell has an increased capacity for arresting cell mitosis when chromosomal DNA is damaged, e.g., by drugs or radiation. Illustrative examples of eukaryotic checkpoint genes include *RAD-9*, *MEC-1*, *RAD17*, *RAD24*, *MEC-2*, and *MEC-3*. The *mec1*, *mec2*, and *mec3* genes were identified in *S. cerevisiae* as mutations that are also deficient for G2 arrest after DNA strand breaks. The gene for MEC1 has recently been cloned, and does not show any homology to RAD9 or other known proteins.

By convention, and as followed herein, terms in capitalized italics refer to the wild-type gene; lowercase italics refer to mutants of the gene; and capitalized nonitalics refer to proteins encoded by the wild-type gene.

"Complementation" is used herein as a genetic term intended to mean that the subject genetic element is homologous to a mutant genetic element such that when introduced into a cell it rescues the cell from the effects of the mutation. For example, *MEC-1* DNA rescues the *mec-1* defect in a *mec-1,cdc9-8* cell (and *RAD9* rescues the *rad9* defect in a *rad9,cdc9-8* cell) through a process herein referred to as

complementation, and the *MEC-1* (or *RAD9*) DNA so capable is referred to as a "complementing cDNA." Similarly, *huCDC34* is homologous with yeast *CDC34* and able to complement the mutation in single (or double mutant) cells, e.g., single mutant cells of yeast strain *cdc34*.

"Suppression," "suppress," "suppressing," and "suppressed" are used herein as genetic terms intended to mean rescue of a mutant phenotype by a non-homologous genetic element that circumvents the effects of mutation. For example, human *CDC34* is not homologous with *mec-1*, (i.e., no significant homology detectable by computer-assisted alignment and sequence comparison), and yet *huCDC34* is capable of rescuing the *mec-1* defect in a *mec-1,cdc9-8* cell. Similarly, human $RAD9_{compA}$ is not homologous with *RAD9* and yet it is capable of rescuing the *rad9* defect in a *rad9,cdc9-8* cell by suppressing the mutant checkpoint *rad9* function.

"G2 arrest" is intended to mean arrest in the interval of the cell cycle following DNA replication and before mitosis. Illustrative examples of ways in which a cell cycle may be arrested in G2 include X-irradiation, gamma-irradiation, ectopside, and other physical and chemical agents.

A highly sensitive and selective yeast temperature-sensitive selection assay system has now been developed for identifying and isolating mammalian checkpoint genes operative in compensating for a defective G2 checkpoint function. The checkpoint genes so identified are operative in G1 arrest and/or G2 arrest. Using this assay human cDNA clones have been identified and isolated that encode human checkpoint proteins that are functionally active in correcting defects resulting from mutations in yeast checkpoint genes. Three representative human checkpoint cDNA clones which suppress for a defective G2 checkpoint function in a yeast mutant have been identified by this process.

An exemplary method for isolating and selecting human checkpoint cDNA clones that suppress a checkpoint mutation in a double mutant yeast test cell is conveniently conducted using the following steps. First, a double mutant test cell is constructed with a mutation in gene #1 that is "conditionally" responsible for DNA damage, and a mutation in gene #2 that causes a growing cell to fail to arrest the cell cycle at a checkpoint where a native (wild-type) cell would arrest if DNA damage was present. The mutation in gene #1 is thus made potentially lethal by the additional mutation in gene #2. "Conditionally," as used herein, is intended to mean that if the mutation in gene #1 is silent the cells grow normally, but when the mutation is induced to become active at a restrictive condition the cells undergo DNA damage. "Restrictive conditions" include, for example, shifting the temperature from a

permissive to a restrictive temperature, or adding an inducer or activator that promotes expression of the DNA damage phenotype of the mutant of gene #1. Yeast double mutant test cells are constructed by mating the respective single mutants of gene #1 and gene #2 and by then selecting recombinants, e.g., using selectable markers. The yeast double mutant test cell has the following desirable phenotypic properties: under permissive conditions the cell grows, but when shifted to restrictive conditions DNA damage results; the mutation in gene #2 prevents cell cycle arresting to repair the damage, and chromosomal aberrations result. Preferably, the double mutant yeast test cells fail to grow under the restrictive conditions, and most preferably the double mutant test cells die when the restrictive conditions are imposed. Illustrative examples of double mutant test cells are provided by *mec-1,cdc9-8* and *rad9,cdc9-8*. In both of the latter test cells the mutation in gene #1 is provided by *cdc9*, which is conditionally lethal at a restrictive temperature; and the mutation in gene #2 is a *mec-1* or *rad9* mutation capable of preventing G2 arrest in response to DNA damage. There are multiple *cdc9* alleles; one preferred allele is provided by the *cdc9-8^{ts}* strain. *rad9* yeast mutants lack a functional RAD9 protein that is essential in yeast at a G2 checkpoint. The *mec1,cdc9-8* or *rad9,cdc9-8* double mutant test cells die more rapidly at 36°C than *RAD9,cdc9-8* or *MEC1,cdc9-8*, respectively. The latter two different illustrative double mutant yeast strains have been deposited: *rad9,cdc9-8* cells as ATCC No. 74154; and *mec-1,cdc8-9* cells as ATCC No. 74155.

Second, pooled human cDNA is inserted into a plasmid vector having a selectable marker under the control of a yeast promoter, and the vector is introduced into cultures of the yeast double mutant test cells, e.g., using lithium acetate transfection. Transformants are selected as individual colonies (based on marker expression and temperature), but *en masse* on microbiological culture plates. Next, tests are conducted to determine the plasmid dependence of the phenotypic expression (e.g., plasmid-dependent growth). In this case the yeast cells are "cured" of the plasmid and then tested for phenotype (e.g., viability or growth). The latter test results are used to ensure that the phenotype selected (e.g., growth or viability) in the transformant is dependent upon the presence of the human cDNA in a plasmid DNA, and not on some other random genetic event. In the illustrative examples it was necessary to isolate about 200,000 transformants so that sufficient transformants would be available for selection of the rare suppressor human cDNA clones.

Third, transformants from step 2 are tested to isolate the few colonies whose phenotype (e.g., viability or cell growth) is not conditioned by the activity of gene #1

(made lethal by the effects of gene #2). In this case the few transformants that are capable of growth have been rescued through the action of a human cDNA that either complements or suppresses gene #1 or gene #2. For example, with *cdc9-8^{ts}* the conditional nature of the double mutant test cells is conveniently determined by assaying cell viability as a function of temperature. The *rad9,cdc9-8* cells have a phenotype of rapid death at the restrictive temperature and less than 1 in 1000 of the human cDNA transformants survived the shift from the permissive temperature (23°C) to the restrictive temperature (30°C). "Suppression" of the double mutant yeast test cell phenotype (e.g., lack of growth and particularly lethality in this example) is intended to mean that the subject human checkpoint DNA increases expression of the phenotype (e.g., viability) of the *rad9,cdc9-8^{ts}* double mutant without complementing either the *rad9* gene or the *cdc9-8* gene, with "complementation" requiring genotypic homology in order to rescue the phenotype.

Fourth, clones isolated under restrictive conditions are considered to be candidate clones for human checkpoint DNAs. Candidate clones are subjected to further phenotypic and nucleotide sequencing analysis to confirm their identity as human checkpoint clones. Three common methods (illustrated in the Examples, below) can be used to distinguish complementing cDNA clones from clones that exert their effects in the double mutant test cells via suppression:

First, cell viability of transformants may be compared with that of double mutant test cells, vector-transformed control cells, and double mutant test cells transformed by the native (wild-type) gene. The comparisons are conducted under different restrictive conditions (e.g., at different temperatures, such as 37°C, 34°C, or 30°C for *rad9,cdc9-8* cells). In the illustrative examples presented below, when complementing *huCDC34* DNA (or the yeast *MEC-1* gene) was introduced into a *cdc34* cell (or *mec-1* cell) the mutant acquired the growth characteristics of the native wild-type *CDC34* or *MEC-1* transformed cells. In contrast, human cDNAs exerting their effects on the double mutant test cells through suppression, rather than complementation, can exhibit distinctive differences in these comparative tests.

Second, growth rates of human transformants may be compared at different restrictive conditions with growth rates of vector-transformed control cells, and double mutant test cells transformed by the native gene. In this case the homolog should theoretically provide greater phenotypic expression than the suppressor cDNA; however, those skilled in the art will recognize that phenotypic expression of genes can be undependable.

Third, confirmation that a human cDNA acts by suppression, rather than complementation, is provided by sequencing the cDNA in the screened transformant (i.e., in the plasmid DNA from the transformant) and determining that the cDNA does not have a nucleotide sequence homologous with either gene #1 or gene #2. In all cases, complementing human cDNA clones are those that have a nucleotide sequence more than 35% and preferably more than 50% homologous with gene #1 (e.g., *cdc9-8*) or gene #2 (e.g., *rad9* or *mec-1*). The three human checkpoint genes isolated are capable of correcting the deleterious effects of a mutant yeast checkpoint gene. While all three clones could correct the defect either by supplying the missing gene product (e.g., complementing with a homologous human gene product), or by substituting the missing yeast mutant checkpoint function with a different phenotypically compensating function (e.g., suppression), only the latter suppression has been observed. All three of the human cDNA clones isolated acted by suppression, and not complementation: i.e., clone #1, *huCDC34* (FIGURE 1), suppressed the defect in *mec1,cdc9-8*; clone #2, $huRAD9_{compA}$ (FIGURE 2), suppressed the defect in *rad9,cdc9-8* and *mec1,cdc9-8* (and is not homologous to *RAD9*); and clone #3, $huRAD9_{compB}$ (FIGURE 3), suppressed the defect in *mec1,cdc9-8* (and is not homologous to *MEC-1*).

Pursuant to the present disclosure, novel checkpoints for DNA repair may be identified through a variety of methods commonly known and used in the art. Similarly, methods are available for selecting novel checkpoint mutants.

DNA repair can result from DNA strand breaks induced by a variety of treatments, e.g., irradiation treatment with chemical agents or errors during DNA replication. Thus, DNA replication mutants are also useful (in place of DNA repair mutants) as sources of cells for constructing the subject double mutant test cells. Representative examples of DNA replication mutants include *cdc2*, *cdc17*, and *cdc13*. Other examples are described in Weinert et al., *Genetics* 134: 63-80, 1993.

The *rad9,cdc9* and *mec-1,cdc8-9* cells are also useful for identifying and isolating other novel human checkpoint genes, e.g., "huX", "huY", and "huZ", that suppress mutant checkpoint functions. These novel human checkpoint genes are, in turn, used to clone mouse genes "*moX*", "*moY*", and "*moZ*" from which mutant genes "mo^X", "mo^Y", and "mo^Z" may be constructed (e.g., by site-directed mutagenesis and screening for a defective "mo^X", "mo^Y", and "mo^Z" checkpoint function in the assay with the *rad9,cdc9* or *mec-1,cdc 9* cells). The mutant DNAs are in turn useful for constructing mutant murine cell lines (i.e., defective in a checkpoint function) in which DNA strand breakage can be induced, e.g., by radiation or drugs. The latter murine

cells with DNA damage and a mutant checkpoint gene are useful for screening to identify novel human compensatory genes, e.g., "*huA*", "*huB*", and "*huC*". These genes may include human homologs of the native *moX*, *moY*, or *moZ* genes, and/or nonhomologous human genes that suppress *moX*, *moY*, or *moZ* without supplying the missing gene product per se. Those skilled in the art will recognize that this process of the invention is useful for identifying natural inhibitors, cofactors, accessory proteins, and dominant negative and positive regulatory genes affecting expression (e.g., genes that encode enzyme inhibitors of X, Y, or Z, dominant negative or positive transcriptional regulators, and accessory proteins, such as cyclins, that modify the function of a checkpoint gene product in the cell cycle). It is considered highly likely that novel tumor suppressor genes (e.g., similar to Rb, the retinoblastoma gene) will be included in the latter group of genes.

The subject human checkpoint DNAs that are isolated through the practice of the methods of the invention are useful in constructing stable test cell lines of yeast, *E. coli*, and mammalian cells that have the subject checkpoint DNAs stably integrated in their genomes. The latter test cells may be used for screening chemicals, candidate drugs, radiation, etc., for their effects on checkpoint gene expression. The subject human checkpoint genes are also useful for altering sensitivity of a cell to radiation- or drug-induced DNA damage. Increasing sensitivity of tumor cells to chemotherapeutic drugs and radiation may be desirable, i.e., to increase the lethality of low-dose radiation or a therapeutic drug. Conversely, decreasing sensitivity of patient bone marrow cells to the drugs or radiation may be highly advantageous, and the effect may be obtained by modifying the activity of a checkpoint gene product. For example, overexpression of a native (i.e., genetic wild-type; nonmutant) checkpoint gene in a cell may increase cellular resistance to DNA damage. In this case, the increased resistance may be achieved by introducing additional copies of the subject genes into a cell. While not wishing to be limited by any particular mechanism, overexpression of the subject checkpoint may confer increased resistance to DNA strand-breaking drugs, by enhancing cellular functions for: surveillance to determine if DNA is broken (i.e., a noncheckpoint gene); stopping or delaying mitosis so that DNA can be repaired (i.e., a checkpoint gene); and promoting DNA repair mechanisms (i.e., a noncheckpoint gene). Methods are provided herein for experimentally discriminating among these three alternatives.

In another illustrative example, decreasing expression of a human checkpoint gene in a cell (e.g., by introducing antisense embodiments of a checkpoint gene into

the cell, or by introducing dominant negative modulators) may increase radiation sensitivity of the cell.

In another illustrative example, overexpression of a checkpoint gene in a malignant cell may be used to uncouple the downstream uncontrolled growth induced by an oncogene- or growth factor-mediated signal transduction pathway. Over-expression of a human checkpoint gene in a cell may be accomplished using drugs that activate the promoter of the checkpoint gene, or by using gene therapy viral vectors to introduce and alter expression of the human checkpoint gene in the target cell.

The invention also provides for diagnostic screening of cells, such as in tumor biopsy samples, to determine the level of checkpoint gene expression and rearrangement as an indicator of sensitivity of the (tumor) cells to DNA damage by radiation or chemotherapeutic drugs. Other uses of the subject checkpoint genes include gene therapy to increase radiation resistance of bone marrow cells (i.e., prior to transplantation into recipients who may need additional radiation or drug therapy; e.g., AIDS patients with malignant lymphoma). Assays are also contemplated for identifying chromosomal rearrangement of human checkpoint genes, e.g., in tumor cells and genetic deficiency diseases. Examples are provided of how FISH (fluorescence *in situ* hybridization) was used to map the far telomeric region of human chromosome 19p13.3. Since telomeric regions in chromosomes (teleosomes) are subject to frequent rearrangement from incomplete DNA replication and telomerase terminal extension, it is thought highly likely that mapping rearrangements of human checkpoint genes may be useful diagnostically for identifying the underlying cause of gene rearrangements in cancer predisposition syndromes and for identifying targets for gene therapy.

EXAMPLE 1

Cloning of a MEC_{comp} and Identification as CDC34

In order for the cloning scheme to be successful, a cDNA source containing an intact checkpoint mechanism was required. The U118 glioblastoma cell line fulfilled this requirement, as shown by the results of experiments in which the cells were exposed to graded doses of cesium-137 gamma irradiation. Twenty-four hours after exposure of a logarithmically growing culture to 900 cGy, there was a clear accumulation of cells in G2 when compared to unirradiated controls.

A *cdc9-8,rad9::HIS3,leu2* strain (9085-8-3) was constructed in order to select for *RAD9* genes. *rad9::HIS3* signifies a deletion mutant of the *RAD9* gene by insertion of the *HIS3* gene. This type of mutation has a very low reversion frequency.

Phenotypic growth characteristics of double mutant yeast test cells and the single mutant cdc9-8 cells are shown in Tables 1 and 2, below.

Table 1

Temperature dependence of growth of mutant strains of *S. cerevisiae*.

	TEMPERATURE[a]		
STRAIN	23°C	30°C	34°C
cdc9-8,RAD+	+++	++	-
cdc9-8,rad9	+++	-	-
CDC+,rad9	+++	+++	+++

a. Log phase liquid cultures grown at 23°C were diluted and spread onto plates containing rich media, after which the plates were incubated at the indicated temperatures for three days. Growth is scored as +++, large colonies, ++, small colonies, -, no colonies/no growth.

Table 2

Temperature dependence of viability of mutant strains of *S. cerevisiae*.

	TEMPERATURE[a]			
STRAIN	23°C	30°C	34°C	36°C
mec1,cdc9-8	100%	<0.01%	ND	ND
rad9,cdc9-8	100%	<0.1%-	ND	ND
cdc9-8	100%	100%	10%	<0.10%

a. Percent viable cells after 3-5 days. ND, not determined.

We also obtained a *cdc9-8,mec1-A401,leu2* strain (171-10-2) for selection of MEC1 function. No deletion mutant of *mec1* was available. The *mec1-A401* allele is a radiation-sensitive *mec1* allele that has effects similar to the *rad9* mutation on the growth of *cdc9-8*.

The experimental design was as follows. Log phase cultures of the yeast test strains grown at 23°C were made competent for transformation by the lithium acetate method. Transformation of the strains was performed with the ADANS vector (control) or DNA from the pooled cDNA library. The transformed yeast was spread on plates with leucine-deficient media at 23°C for 20-24 hours to select for those yeast which had taken up the DNA and allow expression of the cDNA insert. The

plates were then transferred to a 30°C incubator and allowed to grow for five to seven days.

Control experiments in which the *cdc9-8,rad9::HIS3,leu2* strain was transformed with the ADANS vector alone showed that only one in ten thousand LEU+ cells would grow at 30°C under these conditions. The background rate (number of cells growing at 30°C after transformation with the vector alone) for the *cdc9-8,mec1-A401,leu2* strain was one in five to ten thousand and somewhat more variable.

A series of transformations of the cDNA library into the two test strains were performed. Any colonies that grew within five to seven days at 30°C were streaked out for single colonies, and plasmid dependence for growth at 30°C was determined. This was accomplished by growing the transformants nonselectively in liquid culture (rich media at 23°C) and then plating on rich media to allow spontaneous loss of the plasmid. Replica plating to minus leucine or rich plates at 30°C demonstrated whether growth at 30°C required the presence of the plasmid. Plasmid dependence was confirmed by isolating the plasmid from the yeast transformant, amplifying the DNA in *E. coli*, and retransforming the original yeast strains and selecting for growth at 30°C.

After screening approximately two hundred thousand cDNAs for complementation of the *mec1,cdc9-8* strain, there were 15 primary transformants, only one of which (named *171tx6*) showed plasmid dependence for growth at 30°C. Transformation of the *cdc9-8,mec1* strain with *171tx6* DNA revealed that approximately 20-30% of the LEU+ colonies grew at 30°C compared to a control of less than 0.1%. Transformation with a plasmid containing the authentic yeast *MEC1* gene under its own promoter resulted in nearly 100% viability at 30°C. However, the selection scheme could potentially select for human DNA ligase cDNAs, which can complement the mutant yeast DNA ligase. Transformation of a *cdc9-8,MEC+* strain did not show any evidence that *171tx6* was complementing the ligase mutation directly (e.g., the maximum permissive temperature was still 30°C). The growth phenotype of transformants having the *171tx6* DNA, or subclone *171tx61* DNA, are shown in Table 3 below.

Table 3

Suppression of a lethal growth phenotype in *S. cerevisiae* mutants by transformation with *huCDC34 (171tx61).*

		TEMPERATURE[a]	
CELLS	VECTOR	23°C	30°C
mec1,cdc9-8	*control ADANS*	+	-
	171tx6	+	+
	171tx61	+	+
rad9,cdc9-8	*control ADANS*	+	-
	171tx61	+	+

a. Growth, determined by colony assays similar to those presented in Table 1; +, growth; -, no growth.

Due to the manner in which the library was constructed, there were three unique cDNA inserts in the *171tx6* clone. Each was subcloned into the ADANS vector, and only one of these cDNAs was active (*171tx61*) in the complementation assay. Sequence analysis of the *171tx61* cDNA insert revealed a striking homology between *171tx61* and the yeast cell cycle gene *CDC34*, with a 50% perfect conservation of amino acids in the 110 amino acid conserved core. A lesser homology to other members of this family of proteins was also observed. This family of proteins encodes the ubiquitin ligase E2 enzymes that are an integral part of the complex that targets ubiquitin to cellular proteins. Other members of the E2 family include RAD6, UBC4, and UBC5. *171tx61* did not show any homology to the yeast *mec1* gene. Southern blot analysis with this cDNA as probe revealed substantial cross-species hybridization between human, mouse, chicken and *Drosophila* DNA, and a pattern suggesting only a single gene (data not shown). In addition, a single 1.8 kb transcript has been detected in several cell lines by Northern blot analysis (data not shown).

Interestingly, the human *171tx61* nucleotide sequence terminates prior to the carboxy-terminal region of the yeast *CDC34* gene and prior to an Asp rich region that was thought essential for CDC34 protein and in particular ubiquitin conjugating activity. Since huCDC34 protein appears functional in yeast, the results suggest that the COOH terminus is not requisite for enzymatic activity.

In order to determine if *171tx61* was the human homologue of *CDC34*, we obtained a *cdc34* temperature-sensitive yeast strain from Breck Byers. Transforma-

tion of this strain with *171tx61* revealed almost 100% complementation of the *cdc34* mutation, allowing rapid growth of the temperature-sensitive strain at 37°C. See Table 4.

Table 4

Complementation of growth of an of *S. cerevisiae cdc34* mutant by transformation with *huCDC34*

	TEMPERATURE[a]	
VECTOR	30°C	37°C
control ADANS	+	-
171tx61	+	+

a. Growth, determined by colony assays similar to those presented in Table 1; +, growth; -, no growth.

Complementation of the *cdc34* mutation by the *171tx61* cDNA appeared specific for the CDC34 member of the E2 family, as it did not complement the radiation sensitivity of a *rad6* mutant strain. Thus, we consider *171tx61* the human homolog of *cdc34* and renamed it *huCDC34*. Complementation of the *mec1-A401* mutation was unique to the human *CDC34*. Expression of the *S. cerevisiae CDC34* gene from the ADANS expression vector showed no complementation or suppression of the lethal phenotype in *mec1,cdc9-8* (e.g., growth at 30°C) (data not shown). Ubiquitin conjugating enzymes were not previously thought to function at checkpoints in the G2 phase of the cell cycle.

The mechanisms by which overexpression of the *huCDC34* protein results in the complementation of the *mec1-A401,cdc9* strain are presently under investigation. A current hypothesis is that overexpression of *CDC34* results in a slowing of the normal cell cycle, in particular a lengthening of late S or G2 phase(s) allowing the mutant *cdc9* more time to function. Consistent with this hypothesis, the doubling time of the *mec1* strain containing the ADANS control vector was 120 minutes, while that strain containing the *huCDC34* had a doubling time of 160 minutes. (Comparing doubling times is a method by which certain complementing and suppressing human cDNA clones may be distinguished from one another.)

The sequence of the 1374 basepair *tx61* cDNA encoded one long open reading frame of 298 amino acids, which was in frame with the first 14 amino acids of the ADH gene resulting in a fusion protein. Surprisingly, analysis of the translated sequence with the PATMAT homology program revealed a high degree of homology

to the *S. cerevisiae* cell cycle gene *CDC34* and several other members of the ubiquitin ligase (UBC) family. There is 50% perfect homology between tx61 and CDC34 in the 108 amino acids flanking the active site cysteine. Multiple alignment analysis of *tx61* with *CDC34* (*UBC3*), *RAD6* (*UBC2*), and *UBC5* revealed that the human *tx61* is most closely related to yeast *CDC34*, and yeast *CDC34* is more related to *tx61*, than the other yeast members of the family. For example, there is an insertion in the CDC34 (12 amino acids) and tx61 (13 amino acids) proteins between the two highly conserved regions surrounding the active site, which is not found in most other members of the family. In addition, they share a highly acidic carboxy terminal end that distinguishes a subgroup of the *UBC* genes (*CDC34* and *RAD6*) from the other UBC genes. The wheat germ *UBC7* gene is also very homologous to *tx61* but does not have the acidic carboxy terminal end. Interestingly, the human *171tx61* nucleotide sequence terminates prior to the carboxy-terminal region of the yeast *CDC34* gene, and prior to an Asp rich region that was thought essential for CDC34 protein and in particular ubiquitin conjugase activity. Since huCDC34 protein appears functional in yeast the results suggest that the COOH terminus of huCDC34 protein is not requisite for ubiquitin conjugase enzymatic activity.

Southern blot analysis using the human *CDC34* cDNA as a probe revealed specific hybridization to one or a few bands in human, mouse, and hamster genomic DNA. A polymorphic pattern was observed with this probe in different normal human genomic samples confirming utility as an RFLP chromosomal marker. Hybridization to chicken genomic DNA was also detected, as was weak hybridization to *Drosophila melanogaster* DNA, but not to any lower species including *S. cerevisiae*.

Northern blot analysis of several human cancer cell lines reveals hybridization to a unique band of approximately 1.4 kb in length, suggesting that the *tx61* cDNA was nearly full length. Poly A+ RNA from two human neuroblastoma cell lines (SK-M-KCNR and SK-N-AS) and multiple hematopoeitic tumor cell lines was assayed. Human *CDC34* was expressed in all of these lines as expected for a cell cycle regulatory gene, and quantitation revealed only two to fourfold differences among these lines. In addition, RNA from SMS-KCNR cells, which have differentiated and exited the cell cycle after treatment with retinoic acid, showed no decrease in the expression level of *huCDC34* mRNA. SK-N-AS cells that are resistant to retinoic acid also show no decrease in *huCDC34* mRNA expression after treatment with retinoic acid. Thus, no evidence for decreased transcription of human *CDC34* was found when cells were not cycling.

To further characterize the human *CDC34* gene, two overlapping genomic cosmid DNA clones (*34cos2* and *34cos4*) were isolated that are homologous to the human *CDC34* cDNA. The cosmid clones were identified by screening a human placental cosmid library. That these cosmids represented the human *CDC34* gene, and not some other gene, was confirmed by comparison of the restriction map of the cosmids and genomic DNA when probed with the *CDC34* cDNA.

Hybridization by fluorescence *in situ* hybridization (FISH) with cosmid *34cos2* showed positive results in 41 of 42 metaphase human lymphocyte cells examined. The FISH signals were localized to chromosomes 19 at band p13.3 and in the telomeric end of band 19p13.3. One metaphase cell had signals on only one chromosome 19. Hybridization with cosmid *34cos4* demonstrated signals on both chromosome 19 homologs in 38 of 40 metaphase cells examined, and on only one chromosome 19 homolog in the other 2 cells. The signals from *34cos4* were also located at the very telomeric end of band 19p13.3 and were indistinguishable from the signals generated from hybridization with *34cos2*. There was no significant hybridization to any other human chromosomes.

An independent confirmation of this chromosomal localization was obtained by Southern blot analysis of human hamster somatic cell hybrids containing a single normal human chromosome 19. Hybridization with the human *CDC34* cDNA revealed hybridization to the same bands in total human genomic DNA and the chromosome 19 hybrid.

Human *CDC34* cDNA has also been used to map the location of the homologous gene in the mouse genome: the location is at chromosome 11D by RFLP analysis of interspecific crosses using four different polymorphisms. This region of mouse 11 is highly syntenic to human chromosome 17q. Given these results, the genomic DNA from a human chromosome 17 mouse somatic cell hybrid was also probed with the human *CDC34* cDNA, but no hybridization signal was detected (other than that expected for the mouse genome).

The finding that the human homolog of the yeast protein CDC34 complements a *mec1* mutation was surprising. The *cdc34* mutation causes cells to arrest at the G1/S boundary after the activity of START. The arrested cells accumulate multiple buds but do not initiate DNA synthesis. There has been no reason to suspect that CDC34 plays a role in any phase of the cell cycle other than G1, and, given the essential nature of CDC34 at G1/S, there was no reason to look for an effect of a *cdc34* mutation on a G2 checkpoint.

One question from the above results was whether the yeast CDC34 protein (rather than human) might have a compensating effect on the *mec1,cdc9-8* strain similar to that of *huCDC34*. Initial attempts at compensation used a plasmid containing the *S. cerevisiae CDC34* gene under its own promoter showed no compensatory effect on the double mutant cells. To overexpress the yeast *CDC34* gene in a manner similar to the experiment with the *huCDC34*, a fragment of the yeast *CDC34* gene was subcloned that contained the entire open reading frame downstream from an ADH promoter in an ADANS vector. This construct, *scCDC34*, efficiently complemented the *cdc34ts* mutation but very surprisingly had no effect on the *mec1,cdc9* strain for growth at 30°C in numerous experiments.

Further studies were conducted to characterize the interaction of the *huCDC34* DNA with the phenotype expressed by the *mec1* mutant. One model considered that the effect of overexpressing *huCDC34* in the *mec1,cdc9* strain might be nonspecific, e.g., a slowing of the cell cycle by *huCDC34* that allows enough time for the mutant DNA ligase to work. To address this hypothesis, the doubling times of logarithmically growing cultures of *mec1* strains were measured at 30°C in the presence of plasmids. The doubling time was prolonged approximately 30%, i.e., from 2 hours with the control vector to 2 hours and 20 minutes with the *huCDC34*, but FACS analysis of propidium iodide stained yeast cells did not show significant differences in DNA content between the control vector transformed and *huCDC34* transformed cells. Thus, it was reasoned that if slowing the cell cycle by only 20 minutes was sufficient to suppress for the *mec1,cdc9-8* defects, then one might expect that an even less noticeable effect would be seen if cells were arrested for a longer period of time. As a test model, it was found that raising the temperature of a *mec1,cdc9-8* strain to 37°C resulted in rapid lethality with less than 0.1% viability after 6 hours, while a *MEC+,cdc9-8* strain showed much slower loss of viability, i.e., 5% loss in viability after 6 hours. When *huCDC34* transformants were tested in this assay system, it was found, unexpectedly, that *huCDC34* partially restored viability to the mutant *mec-1,cdc9-8* background, even after 6 hours at elevated temperature. Thus, a nonspecific slowing did not appear responsible for the compensatory effects of *huCDC34*.

Interestingly, expression of the *huCDC34* gene does not suppress for the other two phenotypes of a *mec1* strain, namely, radiation sensitivity and hydroxyurea sensitivity. Comparison of transformants, double mutants, and DNA-repair mutants for effects of drugs or irradiation on the cell cycle is another method by which complementing human cDNA clones may be distinguished from compensating cDNA

clones by virtue of phenotypic similarities and differences, respectively. The survival curves are superimposable for a *mec1* strain transformed with a control vector or the *huCDC34* DNA and then exposed to graded doses of radiation. In contrast, a *mec1* strain carrying a *MEC1* plasmid or a wild type strain with the control vector are radiation resistant. Similarly, transformation with *huCDC34* DNA had no effect on hydroxyurea sensitivity (*mec1* strains are unique among the known G2 checkpoint mutations in their exquisite hydroxyurea sensitivity).

The following results support the concept that the effect of the *huCDC34* gene on the *mec1* phenotype is specific: *huCDC34* DNA does not suppress for a *rad9,cdc9-8* strain at 30°C or a *MEC+,cdc9-8* strain at 34°C; and, transformation with the *huCDC34* DNA decreases the lethality of a *mec1,cdc9-8* strain even after 6 hours at the nonpermissive temperature. Surprisingly, the yeast *CDC34* gene does not have the effects that *huCDC34* has on the *mec1* strain. Even when overexpressed there is no effect of yeast *CDC34* on the *mec1,cdc9 8* strain at 30°C . By way of explanation, perhaps the huCDC34 protein is less specific than its yeast counterpart and it is able to ubiquitinate a cyclin during the G2 phase of the cell cycle. In this manner the huCDC34 protein may delay the cell cycle and suppress the lethal phenotype in the *mec1,cdc9-8* cells.

It was also found that although the *huCDC34* gene had a significant effect on the *cdc9* mutant (defective in DNA ligase), it had no effect on radiation sensitivity or hydroxyurea sensitivity of a *mec1* strain. Two additional human checkpoint cDNAs were isolated ($RAD9_{compA\ and}\ RAD9_{compB}$); see Example 2 below. It is proposed that yeast possesses separate mechanisms for creating the *cdc9* checkpoint and the radiation checkpoint, although both pathways must utilize MEC1 and RAD9.

The G1 target(s) of yeast CDC34 is unknown. A possible S or G2 target of human CDC34 is one of the B-type cyclins, CLB1-6, of *S. cerevisiae* that contains a ubiquitin targeting signal. Several of these cyclins have been found to be expressed at a high level in both the S and G2 phases of the cell cycle.

The data maps the location of *CDC34* to the far telomeric region of the short arm of human chromosome 19. The telomeric location of this cell cycle gene in humans is intriguing given the role of telomeric shortening in cellular senescence. A recent model of senescence proposes that repression of essential genes found near the telomere occurs by a change in chromatin structure as telomeres shorten. It is instructive to determine how close to the 19p telomere the human *CDC34* is located, as well as the expression of this gene in cells that are nearing senescence.

In contrast to the human mapping data, mapping of this gene in the mouse places it in a nontelomeric position on chromosome 11D. This region maps to a long region of synteny on human chromosome 17q, but we do not find any evidence for a *CDC34* homolog on human chromosome 17; thus *CDC34* defines a new region of homology between mouse chromosome 11 and human chromosome 19.

This is the first human homolog identified of the group of genes (*CDC34*, *CDC4*, and *CDC53*) required for the late G1 to S transition in budding yeast. Absence of any one of these functions results in cell cycle arrest before DNA synthesis is initiated and the formation of multiple pseudobuds. Identification of the components of the G1 to S transition in human cells will be essential for defining how the initiation of DNA synthesis is regulated and the mechanisms that control the G1/S transition after DNA damage.

EXAMPLE 2

Isolation of Human Genes *huRAD9*$_{compA}$ and *huRAD9*$_{compB}$

A fundamental aspect of radiation resistance is the capacity of cells to detect DNA damage and delay entry into mitosis for a time sufficient to repair the damage. Failure of this mechanism results in unrepaired DNA damage and cell death during mitosis. Both the *RAD9* and *MEC-1* genes play an integral role in the DNA repair surveillance mechanism, and isolation of human *CDC34* as a compensatory cDNA clone for the *MEC1* yeast gene is described above. The experiments described below were designed to identify and investigate human cDNA clones complementing or compensating for the function of the yeast *RAD9* gene in mediating radiation resistance.

Human RAD9$_{compA}$:

In an attempt to clone compensating and complementing *RAD9* cDNAs, approximately 300,000 LEU+ human cDNA transformants of the *rad9,cdc9-8* strain were screened. Forty-five transformants were identified that grew at 30°C. These forty-five transformants were evaluated to determine the plasmid dependence of their growth, as described in the materials and methods below. One transformant (named *83tx42*) was identified that showed plasmid dependence for growth at 30°C. When the plasmid was isolated and retransformed into the *rad9,cdc9-8,leu2* strain, approximately 10% of the colonies grew at 30°C compared to less than 0.1% of the control transfectants. Interestingly, when *83tx42* was transformed into the *mec1,cdc9-8* strain, the cells showed similar levels of growth at 30°C as *83tx42* in a *rad9,cdc9-8* strain. Conversely, *83tx42* had no effect on the *cdc9-8,RAD+,MEC+* strain, suggesting that it did not directly complement the ligase mutation. *83tx42*

contains an approximately 2 kb cDNA insert. Since the cDNA suppressed for the defects of *rad9* in the *cdc9-8,rad9* strain it was termed $huRAD9_{compA}$. The nucleotide sequence of $huRAD9_{compA}$ is shown in FIGURE 2.

Human $RAD9_{compB}$:

In an attempt to identify additional clones compensating and complementing cDNAs, approximately 100,000 LEU+ human cDNA transformants of the *mec1,cdc9-8* strain were screened. Twenty transformants were identified that grew at 30°C. These transformants were evaluated to determine the plasmid dependence of their growth (as described in the materials and methods below). One transformant (named *171tx23*) was identified that showed plasmid dependence for growth at 30°C. When the plasmid was isolated and retransformed into the *mec1,cdc9-8,leu2* strain, approximately 20% of the colonies grew at 30°C compared to less than 0.1% of the control transfectants. Interestingly, when *171tx23* was transformed into the *cdc9-8* strain, the cells showed similar levels of growth at 30°C as in a *mec1,cdc9-8* strain (Table 5, below). Conversely, *171tx23* had no effect on the *cdc9* strain, suggesting that it did not directly complement the ligase mutation. *117tx23* contains an approximately 1.6 kb cDNA insert.

Table 5

Suppression of a lethal growth phenotype in *S. cerevisiae* mutants by transformation with $huCDC_{compB}$ *(171tx23).*

		TEMPERATURE[a]	
CELLS	VECTOR	23°C	30°C
mec1,cdc9-8	*control ADANS*	+	-
	tx23	+	+
	MEC-1	+	+
rad9,cdc9-8	*control ADANS*	+	-
	tx23	+	+
	RAD9	+	+

a. Growth, determined by colony assays similar to those presented in Table 1; +, growth; -, no growth.

The nucleotide sequence of *117tx23* is shown in FIGURE 3. Clone #3 cDNA confers radiation resistance upon both the *mec-1* and *rad9* transformants. For this experiment, the *mec1* or *rad9,117tx23* transformants were exposed to 20 Grey or 60 Grey of X-irradiation; *ADANS* vector transformed *mec1* or *rad9* cells were used as

controls. The clone #3 *117tx23* transformed *mec-1* or *rad9* cells showed 20-fold greater survival after three days of culture.

EXAMPLE 3

Regulation of Human Cell Cycle Genes

Antibodies to the huCDC34 fusion protein were prepared, and antibodies to $RAD9_{compA}$ may be prepared by a similar method. To produce antibodies, the respective cDNAs were subcloned into prokaryotic GST expression vectors designed to produce large quantities of the protein in *E. coli*. The recombinant fusion proteins were used for immunization of rabbits for production of polyclonal antisera. Rabbits showing a positive ELISA response to the fusion protein were boosted with thrombin-treated fusion protein. (The GST region of the fusion protein is thrombin sensitive.) Anti-huCDC34 had an endpoint ELISA titer of 10^4. Antibodies are useful in assays evaluating the level of expression of the *RAD9* and *MEC-1* genes at the protein level. For instance, antisera to huCDC34 and $huRAD9_{compA}$ is useful in Western blot and immunoprecipitation analyses with protein extracts of mammalian cell lines. Such experiments provide information regarding expression of these genes and how post-translational modification, e.g., phosphorylation and glycosylation, may alter expression. In particular, expression assays may be performed in cells before and after irradiation to monitor for changes in the levels of proteins and how changes in checkpoint gene expression correlate with radiation sensitivity or resistance of cells.

To address whether increased expression (overexpression) of a checkpoint gene in a mammalian cell may increase the radiation resistance of the cell, $huRAD9_{compB}$ cDNA was inserted into the pLXSN retroviral vector. The $huRAD9_{compB}$ DNA is under the control of the MuLV LTR promoter. The vector is useful for monitoring changes in G2 arrest and radiation sensitivity of retroviral vector transduced mammalian cells, as compared to that of control pLXN vector transduced control cells.

To show that $huRAD9_{compB}$ plays an important role in radiation sensitivity, $hurad9_{compB}$ mutant mammalian cells are constructed and the mutation is correlated with increased sensitivity of the cells to graded doses of radiation.

Negative selection, i.e., for down-regulation or negative-regulators of *RAD9*, *huCDC34*, $huRAD9_{compA}$, or $huRAD9_{compB}$ function, is accomplished in a yeast screening assay. For example, *cdc13-1* is a temperature-sensitive *S. cerevisiae* cell cycle mutant that causes arrest late in G2 if cells with the mutant genotype are shifted to the restrictive temperature. The maximum permissive temperature for *cdc13*, i.e., the temperature that still allows colonies to form in a yeast with normal RAD9

function, is 25°C. However, in *cdc13* strains in which *RAD9* is also deleted the maximum restrictive temperature increases to 28°C. (Presumably, at 28°C *RAD9* acts on problems due to the *cdc13* mutation and arrests the cells in G2.) In the absence of *RAD9*, the cells continue to cycle and the *cdc13* damage does not make the cells nonviable. Above 28°C, the *cdc13 RAD+* or *rad9* strains do not grow. The result of this effect is that one can select against *RAD9* function by growing a *cdc13,RAD+* strain at 28°C and isolating colonies that can grow at 28°C. Although the difference between the restrictive and permissive temperatures is only three degrees, it has been found that less than one per thousand *cdc13,RAD+* cells will grow at 28°C, as contrasted with growth of almost 100% of *cdc13,rad9* cells. Using such negative selection, two methods are possible for isolating dominant cDNAs that negatively regulate *RAD9*. The first is to randomly mutate the plasmid containing the yeast *RAD9* cDNA. The pool of mutagenized plasmids is transformed into the *cdc13, leu2, RAD+* strain and selected for growth at 28°C. The plasmid from any colony that grows at 28°C is isolated and retested in the same assay. Sequence analysis of active clones is performed to determine what mutation has occurred. The mutant *RAD9* gene is then transfected into wild-type yeast and the changes in radiation sensitivity and G2 arrest determined. The goal is to find a mutant *RAD9* that can interfere with the function of a normal *RAD9* and increase radiation sensitivity. Dominant negative mutants can act by binding the normal protein and forming nonfunctional heterodimers if the protein is normally a homodimer, or by directly interacting with the normal target of the *RAD9* gene.

An alternative approach is to directly mutate a *huCDC34*, $huRAD9_{compA}$, $huRAD9_{compB}$, or *huRAD9* gene and then to transform the *cdc13,leu2,RAD+* strain with pooled cDNAs from a library and select for growth at 28°C. This method allows selection of unique clones which, when overexpressed, may interfere with the function of one or more of the four human cDNAs. Similarly, clones isolated in this manner can be sequenced and tested for their effect on radiation resistance and G2 arrest in wild-type yeast.

Any RAD9 or cDNA clone that has a dominant negative effect on radiation sensitivity in yeast may be subcloned into a mammalian expression vector and transfected into cell lines with moderate to high radioresistance, such as HeLa S3 and U118. Stable lines in which the transfected genes are highly expressed may be isolated along with cell lines containing vector controls, and the radiation sensitivity of the cell lines may be determined and compared. Given that deletion of the *RAD9* gene in yeasts does not affect viability, it is expected that transfectants will be viable.

Changes in cell survival after irradiation and G2 arrest are determined. The goal is to create molecules that actively decrease radiation resistance of tumor cells by interfering with the normal checkpoint function. These molecules represent unique reagents that can decrease radiation resistance *in vivo* and may have therapeutic efficacy.

Materials and Methods

Assay Strategy: A simple genetic assay was developed for selecting human checkpoint genes by complementation of defined yeast mutations. In addition to radiation sensitivity, the presence of a checkpoint mutation increases the lethality of several temperature-sensitive cell cycle mutations. The presence of either a *rad9* or *mec1* mutation decreases the maximum permissive temperature (from 30°C to 25°C) of a strain with a DNA ligase mutation (*cdc9-8*). Presumably, the increased lethality of the checkpoint mutation is a consequence of cells with multiple DNA strand breaks entering mitosis. Thus, after transformation with a human cDNA library, selection for growth at 30°C of a *mec1,cdc9-8,* or *rad9,cdc9-8* strain will allow selection for cDNAs that suppress for or complement the *MEC1*, *RAD9*, or *CDC9* function.

Human cDNA Library: A human cDNA library was obtained in which the yeast expression vector ADANS contained an ADH promoter and first 14 amino acids of the ADH gene flanking the human cDNA insert and the promoter was followed by a LEU2 selectable marker gene. The source of cDNA was the human glioblastoma U118 cell line, which maintains an intact G2 arrest mechanism after irradiation.

Transformation of *cdc9-8,mec-1*: Logarithmically growing cultures of a *mec1,cdc9-8,leu2* strain were transformed with DNA from the cDNA library using the lithium acetate method. The cultures were plated and selected for growth on leucine-deficient media at 30°C. Five days after transformation of the *mec1,cdc9-8* strain with the control ADANS vector less than 0.1% of LEU+ transformants formed a colony at 30°C. Transformation with the human *tx6* cDNA or its subclone, *tx61*, resulted in 10-20% viability of LEU+ transformants at 30°C; for comparison, transformation with the authentic MEC1 gene results in near 100% viability at 30°C.

Yeast and Bacterial Strains: The *S. cerevisiae* strains described in these experiments were congenic with A364a. Sources of the strains are indicated: 171-10-2 (*MATa, cdc9-8, mec1-A401, leu2, ura3, ade2 ade3, trp1-* T. Weinert), *9085-1-8-3 (MATα, cdc9-8, rad9::HIS3, leu2, ura3, trp1), 9085-1-10-4 (MATα, cdc9-8, leu2, his3)*, SJ1098-3d *(MATa, cdc34-2, leu2-3, ura3, trp1* - B. Byers). All bacterial transformations were performed in the SURE strain (Stratagene, La Jolla, CA).

DNAs: A human placental cosmid library in pWE15 was obtained. The *S. c. CDC34* plasmid was constructed by subcloning a PCR amplified 1.0 kb piece of the *CDC34* gene downstream of the HindIII site in the ADANS plasmid. The *MEC1* and *RAD9* plasmids were provided by T. Weinert. Somatic cell hybrid DNAs were obtained from the Coriell Cell Repository (Camden, New Jersey). Both the human chromosome 19 hybrid (GM10449, #5HL9-4) and the human chromosome 17 hybrid (GM10498, #MN-22.6) contain greater than 90% of cells with a single human chromosome, and the chromosome 19 hybrid was negative by Southern blot analysis for a known chromosome 17 marker. The sequence of both strands of the cDNA insert was determined by dideoxy sequencing using Sequenase 2.0 (US Biochemical, Cleveland, OH).

Yeast Transformation: Logarithmic cultures of the indicated strain were transformed according to a modification of the method of Schiestl and Gietz (6), in which the DNA and 50% PEG solution are added directly to the yeast in lithium acetate without any preincubation. Plasmid DNA from yeast was extracted by glass bead disruption and transformed into *E. coli* by electroporation (Bio Rad, Hercules, CA). Plasmid DNA from a single colony was retransformed into the parent yeast strain to check for plasmid dependence.

Northern and Southern Analysis: Total genomic DNA was restricted according to the manufacturer's recommendations and separated on 0.7% agarose gels with TBE buffer. Transfer to GeneScreen *Plus* and hybridization was performed according to manufacturer's recommendations (NEN, Boston, MA). The most stringent wash was 0.2xSSC plus 1% SDS at 65°C. The human *CDC34* probe was a 784 bp PCR product labeled by random oligonucleotide-primed synthesis (Boehringer Mannheim, Indianapolis, IN). The oligonucleotides used to generate the PCR product are 5'-AACACCTACTACGAGGGCGGC-3' and 5'-GCCCGTCCACCGAGCCCCGAG-3'. Poly A+ RNA, a gift of Carol Thiele, was separated on 1% agarose, formaldehyde gels, and also transferred to GeneScreen *Plus* membrane. The filter was sequentially hybridized with the human *CDC34* PCR probe and a rat *GADPH* cDNA. Quantitation of the hybridization signal on the Northern blot was performed by direct phosphorimaging of the hybridized filter (Molecular Dynamics, Sunnyvale, CA).

Fluorescent *In Situ* Hybridization: As described previously (7), posthybridization washes were performed at 42°C and 50°C for 34cos2 and 34cos4, respectively.

Citations

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The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A nucleotide sequence capable of hybridizing under stringent conditions with the *huCDC34* nucleotide sequence of FIGURE 1.

2. A nucleotide sequence capable of hybridizing under stringent conditions with the $huRAD_{compA}$ nucleotide sequence of FIGURE 2.

3. A nucleotide sequence capable of hybridizing under stringent conditions with the $huRAD_{compB}$ nucleotide sequence of FIGURE 3.

4. The nucleotide sequence of claim 1, capable of arresting a cell cycle in a G2 phase in a *mec-1,cdc9* cell.

5. The nucleotide sequence of claim 1, capable of arresting a cell cycle in a G1 phase in a *cdc9,mec1* cell.

6. The nucleotide sequence of claim 2, capable of arresting a cell cycle in an S phase or a G2 phase in a *cdc9,rad9* cell or a *mec1,cdc9* cell.

7. The nucleotide sequence of claim 3, capable of arresting a cell cycle in a G2 phase in a *rad9* cell.

8. The nucleotide sequence of claim 3, capable of conferring radiation resistance to a cell.

9. A method for isolating a human checkpoint cDNA that is capable of restoring growth at a restrictive temperature in a yeast test cell, wherein the yeast test cell comprises a genome having a first gene that forms a DNA strand break at a restrictive temperature and a second gene that fails to induce a cell cycle arrest in response to the DNA strand break, whereby the growth of the yeast test cell is inhibited at the restrictive temperature, the method comprising the steps of:

obtaining a human cDNA library comprising a plurality of human cDNA clones;

inserting the human cDNA clones individually into plasmid vectors comprising a selectable marker gene;

transforming a culture of the yeast test cells with the plasmid vectors from the preceding step;

selecting for yeast test cells transformed with the selectable marker gene;

growing the selected transformants at the restrictive temperature and isolating a candidate transformant capable of growing at the restrictive temperature; and

identifying the human cDNA carried by the candidate transformant as a human checkpoint cDNA by sequencing the human cDNA carried by the candidate transformant and determining that the human cDNA is less than 50% homologous with both the first gene and the second gene.

FIGURE 1. Human *CDC34* cDNA sequence

ATTGGTGAATCCGTCCACTCAGTGCTGGACGTGGCTCCAGGACCTGGAGCTGACA
GGCAGGACCGGGCCCCTCGGACCGCTACACCTGGGCCTCCCAGGCTGGTAGTGTC
AGGAAACGGCCCCCCGNNCACGTTCCCAGCAGCGCCCCCGTGGCTCCTCCGGGGT
GCGGCCAGTCCGGAAGCTGGGGGACCCCGGTAGAAGTCGGGCTCAGCTCCCCTC
CCGAGGGGACAGGTGGGCCGGCCGCTCCCACCCTGGGCCCGTCCACCGAGCCCC
GAGTGACGTGAGTGGCGGTGGGGCAGCCCCTCTTCTCTGAAGCACGTGAAAACCC
AGAACAGACATGGGGAGGGAGAAAAAGCCAAAACGAAACAACCAGAGGAGACG
GGGACCAGCACAAAACCTCCGTGAGGTAGTCTGTCGTCTAAGGAGCCACGGGTCC
GGCCCTAGTGAGGTAAACTCGGCAAGTTTATTCTGGTGGTGTCAGGACTCCTCCG
TGCCAGAGTCATCCTCATCGTCCCCGAAGCAGCTGTCGGCCTCCTCCTCCACCTCG
CCGTCTCGTAGTAGTCGTCGTAGAAGAGGTCTGAGCCCTCGTCGGGCGCCGGCGC
CTTGGTCTTCACGCAGTACTCGGCCAGCGTGGTGGGCACCTTCACGCCGTCACGC
TCCGCGTCCACCTTGGTCCCCAGGACCTGCTTCCGGATGATGTCTGTGTACTCCCG
ATCCTTCCCCTTGCTCTCTTTCCACTTCCTGTACATCACGGAGGCGTCCACGTTTG
CGGGCGAGAAGGTGTTGGGCTCGTTCAGGAGGGAGATCACACTCAGGAGAATGG
TCCTGACGTTCTGCGTGGGGTTCCACCTCTCTGAGGGCAGCTCCCCGCTCTGGGG
GTCGTCCACCGGCGGGTGGAGGATGGAGATACACACGTCCCCCGTCTCGTAGATG
TTAGGGTGCCACATCTTGGTCAGGAACCGAAAGGCTGGTGGAGAGTATGGGTAG
TCGATGGGGAACTTGAGGCGCGCCTTGAAGTAGCCGCCCTCGTAGTAGGTGTTGG
GGGGCCCGAAAATGGCCACCTCCCAGTTGTATAGATCGCCCTCGTCCACCAGTGT
CACGCGGAATCCCTCGACCGGCTCTTCCTGCAGCCCCTTGAGCTCCAGCAGCAGC
GCCTTCTGCGAGCTGGGCACTAGCGGCCGAGCCATGGCGGCGGCGGAGGGGCCC
GGGGTCGGAGCAGCGCGNGGCCGCGCGACCACCGCGAGTTCGCGAGACGGGCCG
GGCCGCGCACCGTCCGGGGGGGGAGCCACCGGGGCCGCCGCCTGCCTCCTCCTC

FIGURE 2. $RAD9_{compA}$ cDNA sequence

ATTGTTCTATTGATGGCAGGTAATCATCACTCTTCACTAGCTGAGCATTCGGTCCA
CTAACCTGAGTCATATCCGGCACTGGTTTCTCTAGAAAGGGNTCCGACGGGGAAT
GCTGATGCACAGGCACTTTCTGCGGGGTGTTCTGGGGTGATGGGTGGAGCTGTGC
CCAAGGCTGGTGATGAGGGTGTGGAGGTGAAGACTGGTGGTGCAAGCCCGGGTG
AGGCTGCAGTGGAGGACAGGTTGGAACTGCTGAAAAGATGGCTGTTGACCAGGA
TGTTGTTGGCCAGGTATCAGTCGTTCCTGGATTGCTTGTGGGTCTCCAAGGCCAA
CACCAGGACAACCATTTGGCCTCATGTGCCCAGTCAATTCCCTTGGTGCCGAGGA
CATGCCTATAAATGGACGAGACTGCTGCATGTTTCTGGGGCCCATATTCCTCTGTC
CGATTCCCATGGCACCAGGGGGCTGGTGAGATGGCTGAGGATGGGGCATATTTG
GATAACTGCCAACTTCCATTGGTATCCCAGCACTTCCCGGCCTGACTTGTGGAGG
AGGAGTGCCTGCTGGATTACTCATTGCTTTCATGGGTGACATGGGAGGTGGAGAG
GCATAAGTTCCCTGAGGCTGTGAAGGATGCATAGTTTGTGTGTTCATTTGGTTAA
GTGAGCCACTGGGGTGGATGGGCTGCTGGTGCATTAGTCCTTGACCACTGTTTGA
TGGGAATCCTACAGCATTGGGGTATCTTGGTACGGACTGATTCATTGGAGTATTA
TTTGTAAGGCCTAAATTTTGATTCATCCCTGTATTGTTAACTAATCCCTGATTTAG
GTTACTGTAAGGATATCGAGAATACTGCCCTGAGTTGTTTATAGTAGGAGAGGGG
ACTGTCTGGCTCCGAGAACTAAAGTTAAGGGTTTGCGGCCTAACAGCCCCTTGTT
GGGGAGGATTCGGGGAGAATCTGGGACTGTGGGCAACGGATTCTCCATGGAGAG
CAGTAGAGGGGTGGTGATGGAACTGCTGCACCGAGTGACGCAAGGAAGGTGCCA
TGCTGGGACTCTGCTGGGGCACGTGGGACAAGTGGCCAGGTCCTGAGGTGGCAA
TAAAAGGATTTCCCTGATTGAGGCCCTCTTGGCCTTGGGAAAACTGGCTCATCCT
CTGCTGTGGCTGACCATGCTGCTGCATGGAAAAATCCCCACGTGCCATATAGCTG
CCCATCTGCTGCATGTGCTGAGGGTGGCCCTGTGCAGGGGGCCCCGACGGAGCCG
GCTGCGGTGGCTGCGGCTGTGGCTGCTGCTGCTGGTAGGGGGCTCGTATCTGGTC
TGGTACCTGAACAGCCCTGGGGCCCCACATGGAGCTGCTGTCCACAAAGGATTGC
CCATGCCTCTCATTCTGCATGCCAGGGTAGACACCCATCTGACCGCCACCACTGC
CACCATGGGGCACCTGAGGAACGGGAGGGGTGTGATACTGCGAGTGCGGAGACG
CGAGTCCGTTCCCAGGGGTGTTGCTCATCATTCTGTTCGGCTGATCCATCAGATGC
ATCTTTTGTTGTTCATACTGATTATAGTGATCAAAATGTGTCAGCTTTGTTTGATT
TTGATTAGTTGAAGGATGATGAAGGGATGGCTGTAAAGAGGCAAAGCCTTGGTCT
ATTGGCATTTGCTGACCCATAGGATTTACTGGATTTTCCGGGTAACCACATTCTCC
GAGGCCTTCAAGACCTTCACTGAAAATATTCCCATCCTCGCCAAAAAGACTCATC
ATTCCTGGATCTGCCATCTTATTCCAACACACGGCTCCTCCAAACCACAGCTCAGG
AGCTTGCCTGTGCTTCACTTCACTGAGGTGTTTGCCCTCAAAGCCTATATGACCAA
TCCCTAATTGCTGTCCTGATGATGA

FIGURE 3. huRAD9$_{compB}$ sequence

ATTGCAGGTTCCGTAGCTTTCTAGTTTTTTTTTTTTTTTCACTTGGATCAAATAGTT
TTGATAGACAGAAAAAGATCTGTACCATTATTTCCTTTCCTTAACAGCTATTGTAA
TTTCCTGGACTTGGTTGCTTTTCACTTGGGCAGTTAAGAAGACACAGCTTGTTTTC
CCCATCAGTTTTCTCTCTCTCTCCTTCGTGTGTGTGTGTGTGTGTGTGTGTGTGTG
TGTGTGTGTGTGCGTGCGTGCACAGGGCCAATCTTCAGGCTTATGGCTTTTGGAA
CATTTTCTTAATTTAATAGAGAACAGAATTCAATGATTAGCAACATCACTAAAAA
TTTACCCCATTTCTTCTCCATGAGTCACTGACACCCGATGCGCATGAACAGTCCAA
CGTCCACCTCGTAAGATGTCATCGGGGTTCAGGGTTCAGAAGCATCGAGGACTGG
TGGCCGGCCCTCTGTGCTCGCCGTGTGACAATTCCAGTGGCTTTCCTGGCACCATC
AGATGCCTGGTGCCACAAGCTTGGGTCTGCTCCTAGGGGGACGAGGGGTTCCTCC
TCCTCCTCAATTGCTTTATGTGCCTTCACTCAGTGAACCCCAATGGGATGGACAAC
CTGACTTTTTAAACCTAAGGGTTGGGCCTGAACGATGATTACTTTGCCCACGTGC
CTTCTAGGTGCCGAATGTGTGTTCCTGTGATATTGACGTTGACATCCCTGCGGATT
CAGCCACAGGTTTCTGACAAGCTGGAGGAAGCAATGGTAATTTTGGCTTTTTCGG
TTTTGTCTTCAGATAATGAAAAGCTTTTGTAAAACAGCTGAGTGTCAATATGAGTT
CTATGGCTTCAATCTCCTTTAAAAATAAAATTCTTAAGGGTCCAAAACAAAGAAG
AGGGGGCAAATTAAAACCCAATAAAAGGAAAAGAAAAGAAAGAAAACCAAACCC
CAAACAAGAAAAAAGAAAAAAAATTGCTGATATTGCCACAAATCATTAGAAATCT
CCTGACATGCTGAAACCAAATGGTCGTAAGTTCAAAACAAATCAGTGACTTGTTT
TTAATTTTTTGTGGTTTCCTTTTGCTCTTTCTGCCCCTTTGCCGTCCGATTGGTGAT
GTTATTCAAACAGGACCGGATCCCTGCTAAGTGCAGGAGGGACCCTGCCGCTTCT
TTCATCTCCTCATCATCGCTCTCGGGGGGCTTTTCGGTGCGTCTCTTTTTGAGGGG
CAGTGTGTCGCTGGGGACCTTCCTGGCCTTGGCGAAGTGCTGGCGCTTCTTGTGC
TGGGATGCGTACCCGCTGTCCCCCAGAGAATCCTTGGGCTCCTTCTGGCTGTGCT
TCCTGTCGTCCTCTTCCGTGTCGCTGGGGCTCTCGTGGCTCCGGAAGCTCCCCTCG
CTGCCCTCGCTGCCCTCCTGGCTCCCCTTGGTGGCAAACTCATAGTGGTCGTCGGC
TGAGGAGGAGGAGGAGGAGATGGAGTCGCTGGTGGGCGAGGTGCTCCGGGCGTT
GGAGGACTTGGCACTGCTGTAGTTGTGATCCTCCTTGGGGTCTCCGCTGACCACT
GGGGAGCCACAAGATGGCTCACTCTCAGTCCGCATCCGGCAGCTGGTGATGCCAT
TCCTCATGGCCGCTGTCACCCCAATGGGAGTGATTGGCA

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/04458

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/68 C07H 21/04
US CL :435/6; 536/23.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Biological Chemistry, Volume 266, No. 8, issued 15 March 1991, A. L. Haas et al., "Ubiquitin conjugation by the yeast RAD6 and CDC34 gene products," pages 5104-5112, especially page 5104.	1
Y	Science, Volume 246, No. 4930, issued 03 November 1989, L. H. Hartwell et al., "Checkpoints: Controls that ensure the order of cell cycle events," pages 629-633, see entire document.	2-9

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/04458

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nature, Volume 327, issued 07 May 1987, M. G. Lee et al., "Complementation used to clone a human homologue of the fission yeast cell cycle control gene cdc2," pages 31-35, see entire document.	1-9